Report for 2003CA39B: Use of bioassays to assess the water quality of wastewater treatment plants for the occurrence of estrogens and androgens

There are no reported publications resulting from this project.

Report Follows

Use of bioassays to assess the water quality of wastewater treatment plants for the occurrence of estrogens and androgens –

INTRODUCTION

Over the past decade, there has been a global concern regarding the discharge of chemicals that have the potential of altering the endocrine system of aquatic organisms into waterways. Entering the aquatic environment via wastewater discharge and other point sources, a variety of compounds may bind to the estrogen receptor of resident biota and elicit responses in the animals similar to those when the organisms are exposed to the endogenous hormone: 17β-estradiol (E2). Receiving waters and effluent of sewage treatment plants contain these chemicals in concentrations that have demonstrated adverse effects on the normal physiology and endocrinology of the exposed organisms.

To detect estrogenicity of water, egg-yoke precursor proteins (vitellogenin-VTG) have been utilized as a whole animal indicator in male egg-laying animals. Its synthesis is under the direct control of circulating E2 in oviparous non-mammal vertebrates during egg production (Wahli *et al.* 1981). VTG synthesis is normally limited to reproductive females. Male and juvenile organisms possess the gene, which remains quiescent under normal conditions, however the gene can be easily expressed upon exposure to elevated levels of estrogens. VTG produced in the liver is eventually incorporated into oocytes to provide a source of metabolic energy for the developing embryo. Since the synthesis of VTG in fish is under the control of estradiol, the induction of VTG in male oviparous animals has been considered a valid biomarker for the exposure to estrogenic chemicals.

In vitro assays have primarily been used to evaluate the estrogenicity of water. *In vitro* assays include ligand binding assays, cell proliferation assays, recombinant receptor-receptor assays, and yeast-based screen assays (YES assays). The YES assay uses yeast cells containing the human estrogen receptor (ER), the estrogen-responsive element, and the *LacZ* gene as a reporter coding for β-galactosidase (Routledge and Sumpter 1996). In order for YES activation, an estrogen receptor ligand must penetrate the cell wall of the yeast and bind to the receptor. Compounds that do not bind the ER, but augment endogenous E2 concentrations would not be detected as environmental estrogens utilizing the YES assay.

RESEARCH PROGRAM

Numerous strategies have been implemented in an effort to ameliorate the adverse effects of insufficiently treated wastewater in eliminate endocrine-disrupting compounds. Two processes that have been commonly employed include enhanced wastewater treatment and wetland treatment. The objectives of this study was to use *in vivo* (rainbow trout VTG) and *in vitro* (yeast estrogen screening) assays to:

1) Investigate the estrogenic potencies of tertiary-treated wastewater, and
2) Evaluate the estrogenic activites of wastewater following wetland treatment

Methodology

Rainbow trout (*Oncorhynchus mykiss*) have been widely used to evaluate the effects of endocrine disrupting chemicals (Jobling *et al.* 1996; Thorpe *et al.* 2000; Ackermann *et al.* 2002; Schwaiger *et al.* 2002). Juvenile rainbow trout (Length:10.5 ± 1.2 cm) were provided by the California Department of Fish and Game Mojave River Hatchery (Victorville, California). After being transferred to the University of California at Riverside, they were maintained in a living stream (Frigid Units, OH, USA) receiving filtered dechlorinated tap water at a flow rate of 5 L/min. The water temperature in the living stream was 14 ± 1°C. The fish were fed Purina rainbow trout chow (St. Louis, MO, USA) at approximately 2% of their body weight every day. Light cycles were at 14:10 h (light:dark). Fish were acclimated to these conditions for at least two weeks prior to exposure.

Water sample collection

The Prado Wetland (33°54" N; 117°40" W) is located in Riverside County, California, USA and consists of 50 shallow ponds that have been utilized to remove nitrogen from water originating from the Santa Ana River, which consists of effluent from tertiary treated wastewater from Riverside County during baseflow conditions (Bachand and Horne 2000). The average flow into the Prado wetland is 80 cubic feet per second. The average depth is 2.5-3 feet and the retention time is 6 days. Water samples were collected at the entrance and exit of the wetland. Water samples from the two sites were collected in 80-gallon Polyvinyl Chloride (PVC) containers in July 2003.

The Green Acres Treatment Plant at the Orange County Water District, Fountain Valley, California, USA treats about 7.5 million gallons of secondary treated wastewater per day from the Orange County Sanitation District (OCSD) using direct filtration followed by disinfection with chlorine. The resultant disinfected water is used for non-portable industrial and irrigation uses in Orange County. The water sample was collected in April 2003 in an 80-gallon container. Since the water sample contained high levels of chlorine (approximately 4 mg/L) and ammonium (approximately 3 mg/L) which were lethal to rainbow trout, sodium thiosulfate was added to dechlorinate the water sample and the water sample was aerated continuously for several days prior to exposure to eliminate chlorine.

In vivo assays

Mixed sexed juvenile rainbow trout were exposed to 9 L of diluted (33%) wastewater samples (3 liters of waste water/6 liters of filtered tap water) in 20 L tanks with aeration in a static renewal system for 14 days. Control fish were exposed to filtered dechlorinated tap water only. Water temperature was maintained at 14 ± 1°C. Light cycle was14:10 h (light : dark). Each treatment (control and exposed) had three replicates with 2-3 fish for each replicate. The water was renewed every other day. Fish were fed rainbow trout chow at 1% of their body weight during the exposure.

In vitro assay (*Yeast estrogen screening*)

One liter of the wastewater samples (from all sampling locations) was filtered over 0.45 and 1.2 um Whatman filters (Clifton, NJ, USA). The water was passed over an Empore SDB-XC extraction disk (Mt. Pleasant, SC, USA). After extraction, the Empore filters were eluted with 30 ml of methanol, with the eluate evaporated to dryness and resuspended in 100 ul of ethanol. The sample was stored at 4°C until use for YES.

The YES assay was performed according to methods previously published (Desbrow *et al.* 1998; Huggett *et al.* 2003). Briefly, 100 ul of the extracted samples or standard concentrations of E2 (10^{-4} – 10^{-14} ng/L in ethanol) were added to 700 ul of a yeast cell suspension (with OD_{600nm} = 0.057) in microcentrifuge tubes. The tubes were incubated with caps open at 30°C for 5 days. The dried sample was resuspended in 100 ul of buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1mM $MgSO_4.H_2O$, and 50 mM 2-Mercaptoethanol, pH 7.0) and 400 ul of 10 mg/ml chromogenic substrate (O-nitrophenyl β-D-galactopyranoside, Sigma, St. Louis, MO). Incubation was carried out at 37°C for 1 hour for color development. Absorbance was measured at 405 nm using a microtiter plate reader (model: *Vmax*, Molecular devices, Sunnyvale, California, USA).

The method detection limit of the YES assay was 1 ng/L. Estrogenic activity of the water sample by YES assay was expressed as E2 equivalent concentrations (EEQs).

E2 exposure

Juvenile rainbow trout were exposed to nominal concentrations of E2 of 0 (control, 1ml ethanol only), 0.5 ng/L, 1 ng/L, 10 ng/L, and 100 ng/L in 10 liter of filtered dechlorinated tap water using the same exposure as in the in vivo assays. Measured concentrations were determined as previously described (Belfroid *et al.* 1999; Huggett *et al.* 2003) with minor modifications. One liter of water sample was taken from the exposure tank and filtered with combined 1.2 um Whatman and 0.45 um Millipore filters (Fisher Scientific, Los Angeles, CA). E2 was extracted with the Empore SDB-XC extraction disk previously conditioned with acetone and methanol at a flow rate of 5 ml/min and the disk was eluted with 30 ml of methanol. The extract was evaporated under nitrogen stream until dryness and reconstituted in 0.5 ml of hexane:acetone (65:35, v:v). The resultant mixture was derivatized with 50 ul of Bis-Trimethylsilyl-Trifluoroacetamide (BTSFA, Sigma, St. Louis, MO) at 60°C for 60 min and then evaporated until dryness under stream of nitrogen and reconstituted with 200 ul of hexane. Detection of E2 was performed using an Agilent Technologies 6890N Gas Chromatography System equipped with 5973 Mass Selective Detector (MSD). The capillary column was HP-5MS 30m x 0.25mm, with 0.25 μm film thickness. The GC conditions were: detector - 290°C; ion source, EI mode; injector - 250°C. Column temperature program started at 80°C, isothermal for 2 min; ramped to 200°C with 20°C/min, followed by 2°C/min to 260°C, and held for 10 min. The mass selective detector was used in Selected Ion Monitoring (SIM) mode. The E2 retention time was 23.771 min and the precursor ion was 416 m/z and product ion was 285 m/z.

Analyte recovery was quantified using water samples spiked with 17β-estradiol. The recovery was 60% with a relative standard deviation of 15%. The method detection limit (MDL) was 1 ng/L. The measured concentration of E2 in the solution for the E2 exposure was between 79.9% and 122% of the nominal concentration except for 100 ng/L, for which the measured concentration was 28% of nominal. Analysis of environmental samples for E2 was not carried out due to matrix interference with the analysis (Todorov *et al*. 2002).

Plasma VTG production in juvenile rainbow trout exposed to E2 showed a concentration-related increase after the 0.50 ng/L nominal concentration (R^2 = 0.99, P < 0.0001. Fig.1). The lowest –observed-effect concentration (LOEC) for VTG induction was 1.21 ng/L.

Determination of VTG levels

After termination of the exposure, fish were euthanized in MS-222 (50 mg/L). Blood samples from rainbow trout were obtained by an incision at the caudal peduncle and collecting the blood exiting the incision. Blood was centrifuged at 3000 rpm for 10 minutes at room temperature. After centrifugation, PMSF (Phenylmethyl sulphonyl fluoride; Stock solution 0.1M) was added to the plasma samples at a final concentration of 1mM. The plasma samples were stored at –80^{o}C until analysis.

Vitellogenin concentrations in the plasma were determined using rainbow trout VTG enzyme-linked immunosorbent assay (ELISA) kit supplied by Biosense Laboratories (Bergen, Norway). All assay procedures were followed according to the manufacturer. Briefly, 96-well polystyrene microtiter plates were coated with the capture antibody using 100 ul sodium carbonate coating buffer (50 mM, pH 9.6) per well and incubated at 37^{o}C for 2 hours. After incubation, the plates were washed 3 times with 200 ul per well with phosphate buffered saline (PBS) (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.3) with 0.05% Tween 20. One hundred and fifty ul of blocking buffer (1% of bovine serum albumin in PBS) was added to individual wells of the plates for 1 hour. Next, 100 ul of blocking buffer containing the diluted standard or plasma samples was added to the wells and allowed to incubate at room temperature for 1 hour. After the wells were washed 3 times with 200 ul of washing buffer, 100 ul of the diluted detecting antibody was added to all wells and incubated at 4^{o}C overnight. Secondary antibody in 100 ul blocking buffer was added to each well and the plates were incubated on an orbit shaker (400 rpm) at room temperature for 1hour. After washing, 100 ul of the color development solution (Ellman) was added to each well and the plates were incubated at darkness for 1 hour. The reaction was stopped by adding 50 ul of 2N H_2SO_4 to all wells. The absorbance was read at 405 nm in a microtiter plate reader. Vitellogenin levels in the plasma samples were calculated based on the standard curve obtained from the relationship between the concentration of the standard rainbow trout vitellogenin and the absorbance (for all cases, standard curves have a $R^2 \geq 0.99$).

Total protein levels in the plasma sample were determined using the method of Bradford (Bradford 1976) using bovine serum albumin as standards (0.25 – 2 mg/ml). Plasma samples were diluted in phosphate-buffered saline (pH 7.4) and transferred to 96-well microtiter plates in triplicates. The absorbance was read at 595 nm.

Vitellogenin levels in the plasma samples were expressed as ng vitellogenin per mg of total protein. Estrogenicity of the wastewater samples was expressed as E2 equivalent concentrations as described for YES above.

Statistical Analysis

All statistical analyses were performed using the Statistical Analysis System package (SAS, version 8.0, Cary, NC) unless otherwise stated. Before analysis, data were evaluated for normality with the Shapiro-Wilks test, and for equality of variance using Levene's test. Since assumptions of normality and equal variance (increased standard deviation of the data because of the use of mixed sex juvenile rainbow trout) were violated, data were not transformed and a nonparametric test (Kruskal-Wallis test or Mann-Whitney *U*-test) was used to test the difference in vitellogenin levels between control and treatment groups. The significance level was set at $p \leq 0.05$. For E2 exposure, dose response curves were generated by SigmaPlot software (SPSS Inc, Chicago, Illinois, USA).

RESULTS

Green Acres Plant Assessment

Juvenile rainbow trout exposed to wastewater from the Green Acre Plant for 14 days had a higher level of plasma VTG than control fish. The increase in plasma VTG levels in the exposed fish relative to the control fish was approximately 49-fold. Based on the concentration-response curve ($R^2 = 0.999$.), the VTG E2 equivalent concentration of this tertiary-treated water was 16.92 ± 16.48 ng/L . YES assays indicated that the tertiary-treated wastewater had a YES E2 equivalent concentration of <1 ng/L.

Prado wetland assessment

Juvenile rainbow trout exposed to 33% of the water entering and exiting Prado wetland for 14 days had elevated levels of plasma vitellogenin compared to control fish ($P < 0.05$). The percentage increase in vitellogenin levels in the fish exposed to Prado water relative to the control fish was approximately 21-fold ($P < 0.05$) for entering water and 11-fold ($P < 0.05$) for exiting water. There were no significant differences in plasma vitellogenin levels in trout exposed to Prado influent or effluent water ($P > 0.05$.). VTG E2 equivalent concentrations of the entering and exiting water sample of Prado wetland were 29.80 ± 28.11 ng/L and 24.34 ± 23.17 ng/L. No significant differences in VTG EEQ were observed between water entering and exiting Prado wetland (Fig. 3). YES assays showed that the water samples from the entering and exiting sites had YES E2 equivalent concentrations of 2.57 and <1 ng/L respectively.

Significance:

Wastewaters from the Green Acres Plant had environmental estrogens which induced vitellogenin in juvenile rainbow trout. Entering and exiting water from the Prado wetland also had an estrogenic activity in trout. *In vitro* activity of tertiary-treated water was 10 times less than *in vivo* activity. Wastewater dominated surface water of the Santa Ana River possessed 10-fold greater *in vivo* activity than YES estrogenicity. These data indicate that wetland treatment within the Prado site may not totally alleviate *in vivo* estrogenic activity and ER-based *in vitro* ligand-based assays may underestimate estrogenic activity.